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II. An Electrophysiological Approach to Salmon Homing

by J. C. Cooper and A. D. Hasler

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II. AN ELECTROPHYSIOLOGICAL APPROACH TO SALMON HOMING

by

J. C. Cooper and A. D. Hasler

Laboratory of Limnology, University of Wisconsin
Madison, Wisconsin 53706, U.S.A.

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ASPECTS OF MIGRATION AND ORIENTATION IN PACIFIC SALMON

Edited by

C. Groot

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I. INTRODUCTION

It has long been known that salmon can return with great specificity to their homestream to spawn. How this "homing" is accomplished is still not completely understood, although there has been extensive research in many areas relating to this problem. There appear to be two well-defined aspects of the homing-migration: orientation in the open ocean, and inshore orientation near and including the final journey to the homestream. During the early stages of their life history, it is believed that salmon can store odor information about their stream (i.e. imprint to their homestream odors) and recall this information during the inshore phase of the spawning migration. This paper will deal only with this second phase of homing.

One of the more recent methods of studying homing is the electroencephalographic or EEG technique. Classically, the term EEG refers to signals obtained from mammals (mostly humans) from electrodes taped to the scalp in conventionally defined places (e.g. on the temporal lobes). These signals are thought to originate from the cerebral cortex. More recently, however, the term EEG has been used for recordings obtained from many types of animals (including fish) with electrodes placed both on the skull and into various parts of the brain. The sole criterion for an EEG recording seems to be that it comes from the central nervous system and contains signals that look similar to the classical mammalian recordings. Other terms, such as EOG (electroolfactogram) and EOBG (electroolfactobulbargram), are sometimes associated with the EEG.

The EEG is a particularly useful tool in the study of olfaction (and therefore homing) because it is a relatively simple technique compared to censusing large numbers of fish and to behavioral studies on salmon. From the viewpoint of a physiologist, the EEG is easier to obtain than single unit recordings from the olfactory bulb, where many small cells (often one micron in size) are extremely difficult, if not impossible, to study.

The EEG technique, as used in the study of homing, consists of paralyzing the animal, removing a portion of the skull and placing an electrode in the olfactory bulb. Various chemical solutions or natural waters are then presented to the ipsilateral naris and the signals recorded on a polygraph. In some cases the test solutions produce a "homestream" response (see below).

The electroencephalographic or EEG technique was first explored as a possible bioassay for individual stream recognition in salmon in a paper by Hara, Ueda, and Gorbman in 1965. Since that time there have been many physiological (Cooper, 1971; Hara, 1967a and 1967b; Hara and Gorbman, 1967; Oshima and Gorbman, 1966a, 1966b, 1968, and 1969; Oshima *et al.*, 1969c; Rappoport and Daginawala, 1968; Satou, 1971; Sutterlin and Sutterlin, 1971; Hara and Law, 1972) and behavioral (Dizon, 1971; Hara, 1972a and 1972b; Oshima *et al.*, 1969a and 1969b; Ueda *et al.*, 1967; Ueda *et al.*, 1971) studies that have attempted to evaluate the significance of these signals. Hara has summarized much of this work (Hara, 1970).

This paper will attempt to critically evaluate the EEG as a research tool for the study of homing. We will refer to work on non-salmonids

where it is appropriate. In addition we will describe in detail what we believe is one of the most significant and exciting applications of the EEG technique with regard to "odor" homing and to the basic physiological question of long-term memory. In the past, the EEG technique has been misused in some experiments because of limitations of the technique and its analysis. Moreover, the responses obtained with the EEG technique do not always correlate with responses obtained with behavioral techniques.

II. THE EEG TECHNIQUE

A. The Characteristics of the Signal

We shall discuss various factors that need to be considered in order to use the EEG technique for experimentation. These are: the type and form of the responses; the chemicals that elicit responses; the physical parameters, perhaps independent of the chemical itself (i.e. pH, ionic strength, and temperature) that influence these responses; and the order of sample presentation.

The characteristic "homestream" response described by Hara et al. (1965) consists of a series of high amplitude evoked waves (10 to 40 Hz) from 50 to 500 microvolts in strength. We shall see that there are additional characteristics of the response to an artificial imprinted odor as compared to a response to other stimuli. These include the lag at the beginning of the signal, duration of the response, and adaptation (discussed in "Imprinting to Artificial Odors").

Two broad classes of EEG responses to stimuli are spindling and inhibition. Spindling is characterized by a marked increase in signal amplitude especially at higher frequencies when compared to background activity. Inhibition is shown by a desynchronization and flattening of the EEG trace. These, then, may be referred to as a high amplitude response or a depression response. These categories parallel those of Sieck (1967).

A second group of factors to be considered in the study of the EEG involve the relationship between responses and after-responses or after-discharges. Four cases of this will be illustrated from experiments by Weber (1970). He took 0.06 M solutions of various salts and observed EEG responses to them (Table 1). High amplitude responses (spindling) are shown by positive numbers, inhibition responses by negative numbers in this table of relative values. First, he observed a response when the sample was introduced and then no response while rinsing out the nares with distilled water (e.g. NaCl, $MnCl_2$, and $MgCl_2$). Alternately he found a response to the stimulus and also a response while rinsing (e.g. $SrCl_2$, $CaCl_2$, and NH_4Cl). Thirdly, there was a depression at the time of sample presentation but no after-discharge (e.g. KH_2PO_4, KCl and H_3BO_3). Similar classes of responses can be seen with compounds other than salt. Sucrose produces a low response, while phenethyl alcohol gives both spindling and after-discharges.

Unfortunately, the category in which a compound apparently falls is not always consistent. For instance, Weber, Oshima, Hara and Gorbman (Weber, personal communication) all report large evoked potentials to NaCl in both adults and juvenile salmon. Cooper and Hasler do not find evoked potentials to NaCl in imprinting studies with artificial odors using adult coho salmon, although Dizon (1971) and Dizon et al. (1973) did for juveniles. The introduction of NaCl did produce remarkably constant background levels of activity. The inconsistency of responses may be explained because Cooper and Hasler worked only with Lake Michigan coho adults. These fish had lived in a total freshwater environment unlike the fresh-salt-fresh water regime of the Pacific salmon. Handwash (water in which the hand has been dipped) does give consistent large spindling in all work reported.

Table 1. Effect of 0.06M solutions on olfactory epithelium of yearling coho salmon; 1 indicates the amplitude of spontaneous bulbar activity. Positive numbers indicates spindling, negative numbers inhibition. After-responses with distilled water are sometimes produced as well (Weber, 1970).

Solution	Amplitude	After-Response
NaCl	5	no
$CaCl_2$	5	yes
$MgCl_2$	5	no
$MnCl_2$	3	no
Sr	3	yes
NH_4Cl	4	yes
KCl	-2	no
KH_2PO_4	-3	no
H_3BO_3	-2	no
Sucrose	1	-
Distilled H_2O	1	-

Table 2. Olfactory bulb responses of five coho salmon to amino acid solutions relative to human hand-rinse. Note that isomers produce different quantitative response. These responses are not directly related to behavioral responses (Weber, 1970).

Solution	Solution concentration (Molal)	Average response and range in percent
Human hand-rinse	Unknown	100 ± 12
L-serine	1.3×10^{-3}	53 ± 6
D-serine	"	23 ± 13
L-valine	"	23 ± 10
L-isoleucine	"	23 ± 10
L-cystine	1.0×10^{-4}	26 ± 21
L-methionine	1.3×10^{-3}	113 ± 5
D-methionine	1.3×10^{-3}	4 ± 4

Bulbar responses can be seen with other chemicals, such as amino acid solutions as shown in Table 2 from the work of Weber (1970). Here it is interesting to note that while L-methionine gives higher EEG responses than handwash, it does not elicit an avoidance response in behavioral studies on adult salmon, as does handwash. Therefore, the EEG responses are not always directly related to behavioral responses. It is clear, too, that optical isomers of the amino acids give different responses. L-serine (the active component in handwash) elicits a stronger response than D-serine, while L-methionine gives a vastly greater evoked potential than D-methionine.

Hara (1972b) has recently looked at responses of rainbow trout to amino acids. His results correlate well with the work of Weber. The order of magnitude of the response to the eleven most effective of forty-nine amino acids tested were: L-glutamine > L-methionine> L-leucine > homoserine > L-asparagine > L-alanine > L-cystine > L-cysteine> glycine > L-serine > L-histidine.

Sutterlin and Sutterlin (1971) have also described electrical responses of the olfactory epithelium to various amino acids and to other substances. We feel the use of their data in relation to the olfactory bulb is permissible since they state that compounds excitatory to the epithelium generally resulted in EEG evoked potentials. They provide a list of non-stimulatory compounds as seen in Table 3. Unfortunately for an understanding of the EEG, they could find no satisfactory explanation as to why some compounds are stimulatory and some are not.

Table 3. Non-stimulatory compound tested on the olfactory epithelium. All compounds were tested at 10^{-3}, 10^{-4}, and 10^{-5} M (Sutterlin and Sutterlin, 1971).

Propionic acid	Dimethylamine
Valeric acid	Trimethylamine
Caproic acid	n-Butylamine
	sec-Butylamine
Sucrose	1,4-Diaminobutane
Lactose	
Dextrose	Ethanolamine
	L-2-Amino-1-propanol
Methanol	3-Amino-1-propanol
Ethanol	L-Dimethylamino-2-propanol
Propanol	3-Dimethylamino-1-propanol
Butanol	L-2-Amino-butanol
Pentanol	D-2-Amino-1-butanol
	5-Amino-1-pentanol
Morpholine	

Variations in EEG responses to solutions differing in pH have been found. In a preliminary experiment, (Cooper, 1971) it was found that pH changed responses as shown in Figure 1. In this experiment 0.01 M sodium bicarbonate buffer was adjusted to different pH values with HCl and NaOH. It was found that acid pH 4 and 5 and basic pH 8 and 9 elicit higher responses than does neutral pH 7. However, these solutions also varied as to the concentration of sodium, chloride and sulfate ions as well. To control for the ionic strength of these chemicals, Dizon and Cooper (Dizon, 1971) designed another experiment. A series of solutions were prepared in which NaCl was added to the basic solutions (which had been titrated with NaOH) to balance the extra Cl^- in the acid solutions (from titration with HCl) and to balance extra sodium added with the basic titration (NaOH). The results of this experiment are not clear. There appears to be a maximum in response around pH 7.8. Acid solutions elicit greater responses than do basic solutions (see Figure 2). Although responses to the bicarbonate-carbonate solutions are complex and sensitive enough to indicate a pH sensitivity, the exact shape of the stimulus-response curve is somewhat obscured. This experiment was repeated in the fall of 1972 on spawning salmon and again the results were variable.

Another factor in the EEG method, the ionic strength of the solution, also influences the EEG response to various solutions, but this relationship has not been clarified (Weber, personal communication; Dizon, personal communication).

Another physical characteristic of the solution that influences the EEG has been studied by Dizon (1971). He used a 0.06 M NaCl solution to test how the strength of the evoked potentials changed with changing temperatures of white bass (Morone chrysops) obtained from Lake Mendota. The temperature of the sample was monitored with a thermistor in the cannula that was used to deliver the sample. The altered temperature samples (changed by cooling) were compared to a response to the samples at a reference temperature of 18°C. Figure 3 shows that there is a definite temperature sensitivity in the EEG response. This EEG finding correlates well with the results of behavioral experiments on temperature sensitivity of the olfactory rosette (Dizon, 1971).

The placement of the electrode in the EEG technique must be considered. Although many workers using the EEG technique feel subjectively that electrode placement is not a major factor in determining the EEG response in fish (Weber, personal communication; Satou, 1971), others have reported definite changes in the EEG with electrode placement for avian species (Green et al., 1962; Hernandez-Peón, 1961; Hughes, 1966; Mozell and Pfaffman, 1954; Valverde, 1965). Thus, this factor should be considered, especially when one attempts to use a sophisticated analysis of the data (e.g. power spectra by computer) to look at differences in EEG responses.

The order of sample presentation and the number of times the sample is presented are also of interest. Oshima (Horrall, personal communication) and Weber (personal communication) have reported that the amplitude of the evoked potential to a stimulus appears to change depending on the number of times this sample is presented (with a distilled water rinse in between each presentation). They refer to a "priming" effect where the first time the sample is presented, there is a low amplitude response, while later trials elicit higher amplitude responses.

B. Analysis of the EEG Signals

Most EEG work with fish has been analyzed by taking the integration of the response (i.e. the total area under the curve with the signal as the upper bound and the spontaneous EEG level as the lower bound) and then finding the area under the line generated by this integration. This analysis uses approximately a double integral of the evoked potential. It was hoped that the resulting measure would bear some linear relationship to the magnitude of the response. In a subjective way, it is clear that the greater the response, the greater the area under the integration. Ueda, Gorbman, Hara, and Oshima all used a Grass 5P3 preamplifier which has a finite time constant so that one does not obtain a true integration for many recordings. Use of the Grass 7P10 preamplifier, which has an unlimited time constant, eliminates this problem. More recently, workers in our laboratory have measured the slope of the line of the integration record. It can be shown that for a constant "Y" component (such as a constant travel distance for the polygraph channel), the slope is directly related to the area under the curve.

More sophisticated attempts to analyze the data have met with some success (Satou, 1971; Ueda et al., 1971). Ueda et al. working on Himé salmon (land-locked sockeye) found that temporal changes in each component within the bulbar response were characteristic of the stimuli of different stream waters. These results indicate that each stream has its own characteristic odors and that Himé salmon can discriminate between these odors.

The peak frequencies in the response are also of interest, although no work has been done on fish in this regard. Hughes and Mazurowski (1962) used a heterodyne wave analyzer on the EEG spectra from monkeys given a wide variety of odorants and found that each odor could be reliably differentiated on the basis of peak frequencies. Such differences should be sought in salmon EEG's.

A lack of precision in the analysis of the EEG is exactly why some uses of the technique are inappropriate, when more sophisticated analyses are not attempted. For example, it is impossible on the basis of the EEG response alone to differentiate between strongly aversive stimuli (such as handwash and bearpaw wash) and odors which appear to be attractive (such as egg rinse). In addition, when the applicability of an EEG analysis in defining chemical constituents of homestream water responsible for homing of coho salmon was evaluated, alterations in that water which made it more stimulatory (containing perhaps more of the homestream odor), could not be distinguished from alterations that made the water more aversive [containing perhaps handwash contamination (Cooper, 1971)].

The validity of the EEG technique for determining chemical constituents of homestream water was also questioned because of the variable response of coho salmon to any treatment of the homestream water (Table 4). It is doubtful that this technique as reported and analyzed in the literature can serve as a reliable analytical tool for isolation and identification of the chemical constituents in homestream waters. Again, it is possible that the use of a more sophisticated analysis would have helped in this regard.

Table 4. Variation in responses to replicate samples of raw stream water and stream water stored at 5°C. Mean and the standard deviation of the responses, expressed as the percent of a response to NaCl, are shown (Cooper, 1971).

	Raw	Sample 5°C
Coho 144	66 ± 9	50 ± 25
Coho 146	46 ± 10	32 ± 4

More complex analytical tools such as power spectra analyses with a computer program may help in the analysis of EEG records in some cases. However, it may be that for many comparisons, such as the one to be described later in "Imprinting to Artificial Odors," a visual inspection of the data and an integration of the area under the evoked potential, are sufficient and adequate for this application of the EEG technique.

C. The Effects of Flaxedil and Other Anesthetics on EEG Records

The anesthetic used in an experiment can have a large effect on its outcome. Essentially all salmon EEG's reported in the literature have been conducted with fish paralyzed with flaxedil. The average dose has been 2 mg/kg body weight.

Since flaxedil (gallamine triethiodide), a synthetic curare-like compound, is a quaternary ammonium compound, it was long assumed that it did not cross the blood-brain barrier. All central action has been disputed (Bovet and Longo, 1953; Smith et al., 1947). However, some action on the central nervous system was later noted as a prolongation in the after discharge of the cat after stimulation (visual or auditory) while paralyzed by flaxedil. Straw (1968) claims that this is due to peripheral action on the EEG, while Halpern and Black (1967) claim that it is due to central action. Gellhorn (1958) found a change in the EEG of the cat under flaxedil. Direct effects on the cuneate nucleus of the cat were found by Galindo et al. (1968).

Alternately, in clinical studies, Munroe et al. (1966) found that there is no stoppage of sensory inflow during operations on humans. Holstein et al. (1969) and Buchwald et al. (1969) reported no effect on the auditory sensory system under flaxedil.

In the absence of conclusive data, flaxedil is still assumed to have no significant effect on the central nervous system with regard to fish. Nevertheless, Galindo et al. (1968) recommend the use of succinylcholine, since there are no known effects on the central nervous system or the EEG by this substance.

Many studies on the salmon EEG have used MS 222 (Tricaine Methanesulfonate) to quiet the experimental animal. Since adult salmon can be handled without this substance, and because there have been reports that MS 222 influences sensory systems and the central nervous system, its use should be discouraged.

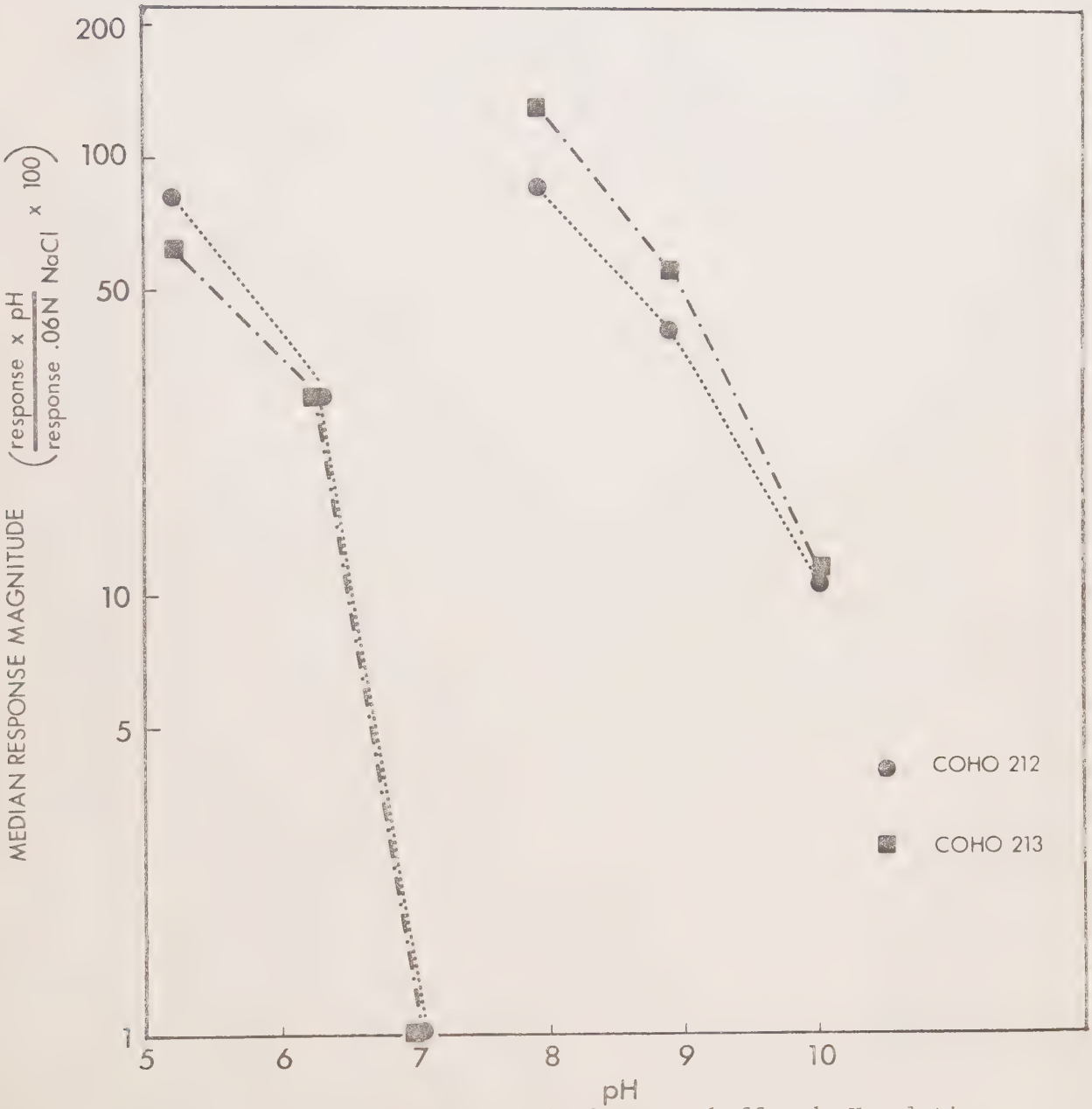

Fig. 1. Responses of coho salmon to different unbuffered pH solutions (Dizon, 1971).

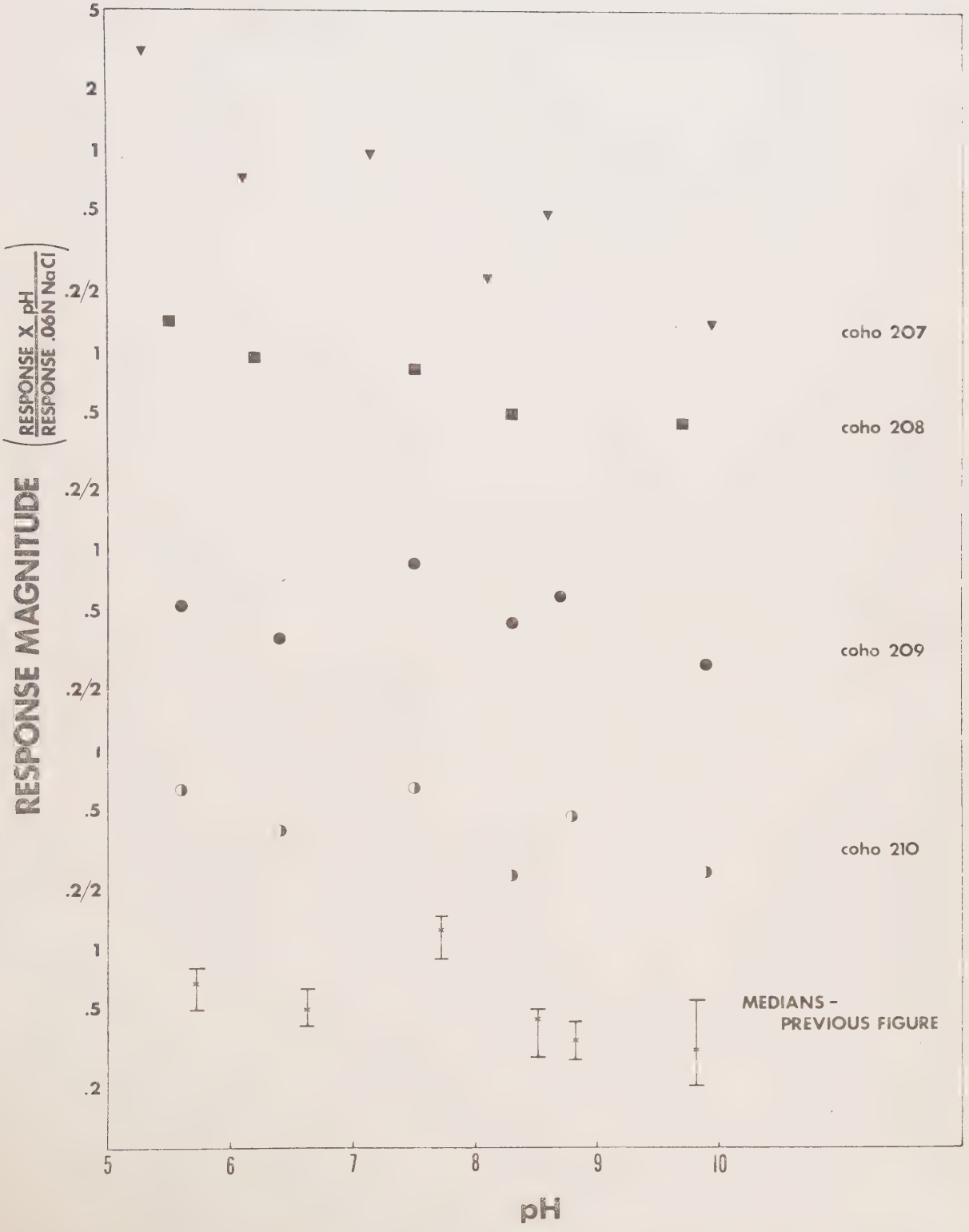

Fig. 2. Responses of salmon to buffered pH solutions (Dizon, 1971).

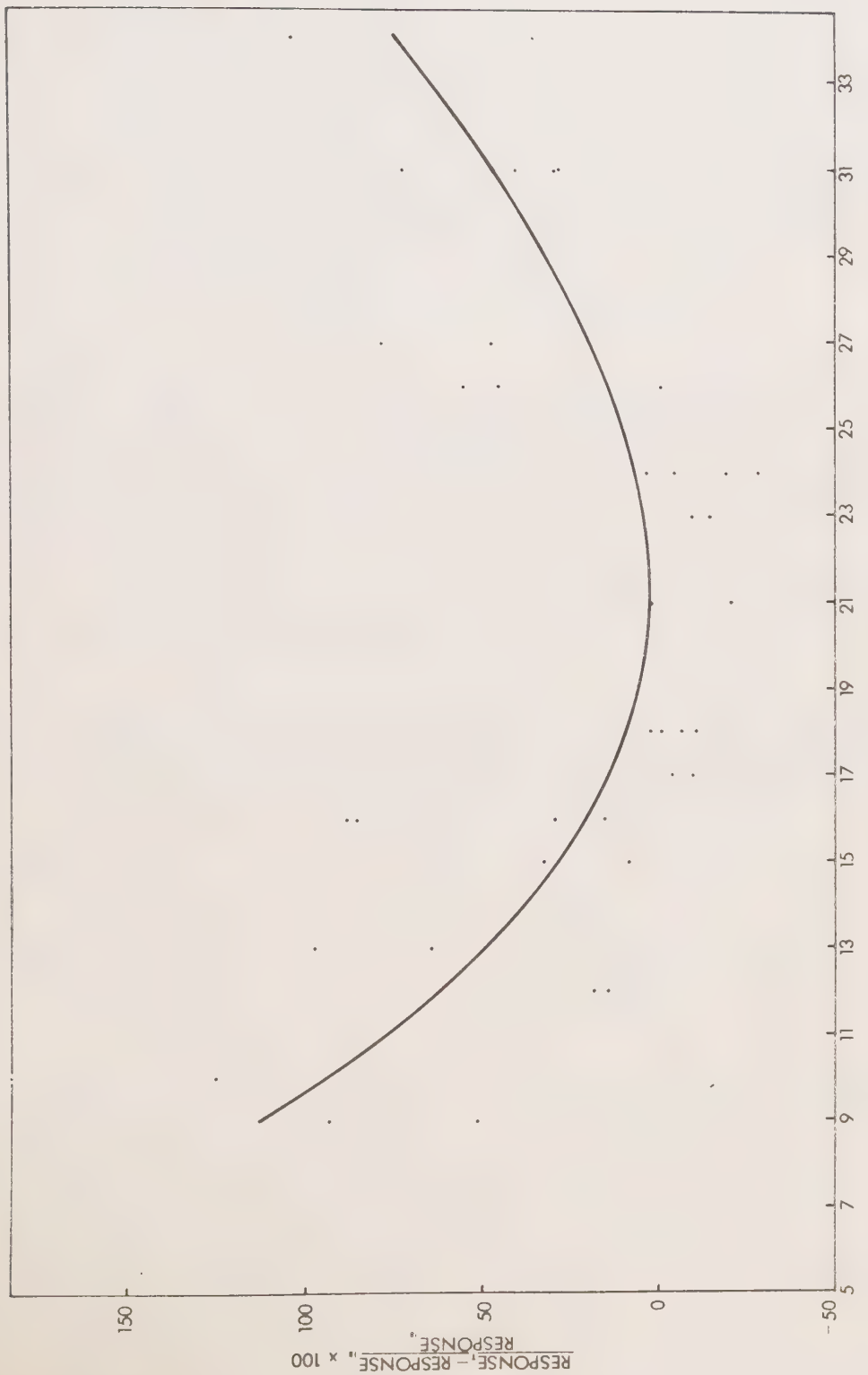

Fig. 3. Responses of white bass to solutions of different temperatures (Dizon, 1971).

III. EEG EVOKED POTENTIALS AND SALMON HOMING

In considering the EEG as a bioassay for homestream recognition, is the evoked potential specific in that only the homestream water elicits an evoked potential? The answer to this question appears to be "no" - other non-natal waters also produce evoked responses. During the discussion concerning this conclusion, one should keep three factors in mind: 1) long-term memory by the adult of its juvenile "imprinting" experience, 2) recent exposure of the adult to the homestream water and 3) incidental stimulatory products in the homestream (Cooper and Hasler, 1972). Since many of the EEG experiments reported in the literature were not adequately controlled for one or more of these variables, the interpretation of the data may need revision.

A. Long-term Memory by the Adult

Earliest reports by Ueda et al. (1967) showed that only homestream water produced large responses in the salmon studied (Table 5). Oshima et al. (1969b) continued these experiments and confirmed the findings, except that they found one non-homestream water that produced large responses in the spawning salmon. The work of Oshima, Hara, and Ueda was conducted on unmarked salmon, which, of course, could not be positively identified as to natal stream. Undoubtedly there was some straying in the groups of salmon they studied, although the extent of this is not known. The terms "natal" or "non-natal," therefore, could not be verified for specific salmon.

Table 5. Specificity of response to home water for salmon from three spawning systems. The magnitude of the responses is represented as a percentage of that to home water (mean ± SE%, number of fish in parentheses (Ueda et al., 1967).

	Water from		
Fish from	Univ. Wash. Fisheries pond	Issaquah Cr.	Soos Cr.
Univ. Wash. Fisheries pond (chinook)	100 (4)	0 (4)	2.3 ± 3.1 (4)
Issaquah Cr. (coho)	39.1 ± 14.5 (3)	100 (3)	6.4 ± 3.5 (3)
Soos Cr. (coho)	0 (4)	0 (4)	100 (4)

Three recent experiments (Ueda et al., 1971; Oshima et al., 1973 ; Dizon, 1971) have failed to find specificity in the response. In the work of Ueda et al. (1971) spawning Himé salmon upon their return to Shimizu Creek or to Senju-Shimizu Creek, responded less strongly to their home water than to the non-homestream water from Jigoku Creek. Similar results were also found in Soos Creek coho and the University of Washington chinook salmon, in which bulbar responses evoked by the home water could not be distinguished from responses to non-natal Issaquah River water (Oshima et al., 1969a). Chum salmon from the Tsugaruis River showed greater responses to non-natal Otsuchi River water than to the home water (Ueda et al., 1971).

Dizon experimented with coho trapped at two different homestreams - the Ahnapee River and the Kewaunee River. The Ahnapee River fish responded to Ahnapee River water but not to Kewaunee water. Kewaunee River fish also responded to their own river water but responded even more strongly to Ahnapee River water. In addition, he found that water in which the fish had been held was more stimulatory than the "pure" water.

In another experiment Tarby (Oshima et al., 1973), working in Japan, found that certain water samples along the homestream produced far greater responses than did other home waters.

B. Non-specific Stimulatory Products in the Homestream

Ueda et al. (1971) have suggested that large responses to non-homestream water may be produced by certain non-specific odorous substances that were merely more highly concentrated in certain non-homestream waters, such as Jigoku Creek. Dizon found that odors from other fish produced stimulatory water samples. This reaction could be considered the result of incidental stimulatory products. Tarby (Oshima et al., 1973) found that the water sample that produced the largest evoked potentials came from a factory discharge and contained large concentrations of organic materials.

The findings reported in A. may be due in part to a problem of water sample collection. Researchers may not have collected "pure" homestream water, but have obtained samples in an area where there is a high incidence of non-specific stimulatory products, e.g. many juvenile or spawning salmon were present.

C. Recent Exposure of the Adult to the Homestream Water

It is pertinent at this point to note that all the work reported here was done with salmon caught near or in the home water. It has been argued that the EEG response merely reflects the odor of the water to which the fish have been most recently exposed and does not indicate a long-term memory of the homestream. There is evidence to support this hypothesis, as seen in Table 6. Chinook salmon taken from Soos Creek water to aquaria at the University of Washington, College of Fisheries, developed a strong response to this new water after being held in it for 67 hours.

Table 6. Olfactory bulbar responses of two-year-old chinook salmon moved from Soos Creek to the University of Washington Fisheries. After only 67 hours responses to the new water is indistinguishable from the responses to the Soos Creek water. (A) vs. (B) not significant; (B) vs. (C) $P < 0.025$; (C) vs. (D) not significant (Oshima et al., 1969b).

Time after transfer (hr)	No. of fish	Bulbar EEG response ratio $\frac{\text{Univ. Wash. Fisheries}}{\text{Soos. Cr.}} \times 100$
0	3	11.1 ± 1.1 (A)
17 - 22	5	62.0 ± 6.3 (B)
67 - 70	3	123.6 + 10.4 (C)
91 - 93	2	117.8 ± 1.6
13 days	3	107.8 ± 9.8 (D)

Evidence against the importance of recent exposure has been found at Oak Creek by our research group. Salmon held in Oak Creek water did not respond to Oak Creek, nor did fish held in a tank supplied with Lake Michigan water show an evoked potential to Lake Michigan water. Moreover, non-imprinted fish (see Section IV) did not respond to morpholine, although they had been exposed to it in the stream. Findings of Tarby (personal communication) in his work with Oshima in Japan, also weigh against accepting the recent exposure hypothesis. We demonstrate in the research reported in "Imprinting to Artificial Odors" that recent exposure is not a major factor.

D. Behavioral Evidence for the Recent Exposure Hypothesis

Behavioral observations for accepting the recent exposure hypothesis comes from a series of experiments we conducted with a Y-maze at the Laboratory of Limnology and Oak Creek.

In our study site at Oak Creek, South Milwaukee, Wisconsin, a Y-maze of plywood was built. Lake Michigan water was put in one arm and Oak Creek water in the other. Salmon jacks held in Oak Creek water for one week always chose the Oak Creek water side of the maze, while fish held in Lake Michigan water for one week always chose the Lake Michigan side (Table 7) (Work conducted with R. Smith).

Table 7. Choices in a Y-maze of salmon held in either Lake Michigan (L. M.) or Oak Creek (O. C.) water for at least 25 hours. L. M. water was delivered to one arm and O. C. water to the other during the experiments. The numbers represent the choices of 8 individual fish run in duplicate (switching the side of O. C. and L. M. water) (with R. Smith).

Held in	Choice	
	Lake Michigan	Oak Creek
Lake Michigan	5	0
Oak Creek	0	3

In a second set of experiments at the Laboratory of Limnology with the same apparatus, Madison city tap water was put into both arms (Steffel and Cooper, 1972). Hatchery-raised coho salmon were held in morpholine at 10^{-4}mg/l for three time periods (12, 24, and 36 hours) in 50 gallon aquaria. When placed in the Y-maze, fish held for more than 24 hours showed increased swimming activity when morpholine was put into one of the arms at 10^{-3}mg/l.

In a third set of experiments (Steffel, 1972; Steffel and Cooper, 1972), juvenile fish unexposed to morpholine showed an avoidance for it when placed in the maze, and swam up the arm not containing morpholine. Fish that were exposed to morpholine at 10^{-5}mg/l for more than 12 hours showed positive responses when they were placed in the maze and swam up the arm containing morpholine at 10^{-5}mg/l. Fish that were held in morpholine for 24 hours were placed in fresh water in order to see the extinction curve to this "memory." These fish again showed negative responses to morpholine after 24 hours in fresh water (Table 8).

Evidence reported by Madison et al. (1973a) also weighs against the recent exposure hypothesis. They found that adult salmon that had been exposed to morpholine in Oak Creek did not respond to this chemical when it was placed in another stream. Other behavioral evidence for and against this hypothesis has recently been reviewed by Scholz et al. (1973).

Table 8. Choices of coho salmon in a Y-maze. The numbers in the left-hand column indicate the numbers of hours the fish was held in morpholine and the time the fish was held in fresh water before the experiment, i.e. 12-24 fish were held for 12 hours in morpholine and then were held for 24 hours in fresh water. D means the fish remained at the intersection of the Y; +, attraction, -, repelled, 0, unaffected. Each symbol in the three right hand columns represent one fish, i.e. there are three fish in each experiment. Groups 36-24 and 12-3 day both have only two fish due to mortality. Columns 3 and 4 are replicate experiments in which the morpholine was put either in arm 1 or arm 2.

Fish not held in morpholine were repelled by it, while fish held in it were attracted by morpholine; the fish were again repelled by morpholine after 3 days in fresh water (Steffel, 1972).

Group	Treatment		
	No Morpholine	Morpholine (Arm 1.)	Morpholine (Arm 2.)
0-0	0 0 0	- - -	- - D
12-0	0 0 0	+ 0 0	+ 0 0
36-0	0 0 0	+ + 0	+ + 0
12-24	0 0 0	- D 0	D D 0
36-24	0 0 0	D 0	D D
12-3 day	0 0 0	- D	- -
36-3 day	0 0 0	- - -	- - -

Table 9. Experimental results of Crystal Springs imprinting. E designates subjects imprinted with morpholine at a concentration of 10^{-5} mg/l; C designates were controls. Median EEG response of each fish to 1% morpholine and 0.01% morpholine stimuli are ranked and Mann-Whitney U values and probability values are assigned to each treatment level (Dizon, 1971).

1%

Group	C	C	C	C	C	C	E	E	E	E	E	C	E	E
*Median	63.5	100	140	220.5	230	250	255	266.5	287	333	351.5	600	915	917
Rank	1	2	3	4	5	6	7	8	9	10	11	12	13	14

$U = 5$ $\quad p = 0.006$

0.01%

Group	C	C	E	C	C	C	E	E	C	E	E	C	E	E
*Median	0	0	0	0	0	25.6	38.5	41.5	45	45	50	50	67	77.5
Rank	3	3	3	3	3	6	7	8	9.5	9.5	11.5	11.5	13	14

$U = 11$ $\quad p = 0.049$

$$\text{*Median Response} = \left(\frac{\text{response morpholine}}{\text{response .06 N NaCl}} \times 100\right)$$

IV. IMPRINTING TO ARTIFICIAL ODORS*

A. Introduction

As noted in the beginning of this paper, it has been hypothesized that salmon can retain the olfactory information about their homestream and use it to find their natal stream during the homing migration. We have used the EEG technique to determine whether coho salmon fingerlings can be "imprinted" (by exposure) to an artificial chemical (morpholine), and to see if they can retain the odor information from this chemical until the adult spawning migration 18 months later. We have found a difference in the evoked responses of previously exposed (as juveniles) versus unexposed adult coho salmon to morpholine during the spawning season. Thus, it is shown for the first time that homing salmon can retain odor information of an artificial chemical affiliated with the homestream for at least eighteen months. These results correlated well with behavioral evidence recorded in a joint study (Madison et al., 1973a and 1973b).

Morpholine was chosen as the imprinting chemical by Wisby (1952) because of certain characteristics. It is infinitely water soluble and, as far as is known, is relatively stable in natural waters. It is not known to occur in natural waters or to be affiliated with any natural stream system. This chemical is also repellent to naive salmon at concentrations several orders of magnitude above threshold, which is very low (about 10^{-6}mg/l).

This problem of imprinting was first approached by Dizon (1971) and Dizon et al. (1973) electrophysiologically. Two thousand five hundred coho salmon smolts were divided into two groups. One group was exposed to morpholine at 10^{-5}mg/l for one month and the other (control group) was left unexposed. The fish were held for ten months and then tested with the EEG technique for their response to morpholine at various concentrations. The responses were integrated and this integration compared to the integration of a response to a standard solution of NaCl (NaCl, as noted earlier, produces consistent evoked potentials and is, therefore, a useful standard). These results were ranked according to the strength of the responses (Table 9). Two of the chemical concentrations (1% and 0.01%) evoked high potentials in the exposed versus the unexposed salmon. Responses of the two groups to 0.1% morpholine were not statistically different. This was the first evidence that the EEG might be used as a tool to study imprinting. However, since the salmon were held in a hatchery after chemical exposure and were not sexually mature, we felt that it was necessary to determine whether adult salmon also showed this response.

B. Experimental Design

Three groups of 8,000 coho salmon fingerlings were stocked into large holding tanks at Oak Creek, South Milwaukee, Wisconsin, in April 1971. These salmon were supplied with water pumped from an intake about 1 km out from the shore of Lake Michigan. This was considered a neutral or "background" water in that it was not associated with any stream system. All of the fish groups were marked uniquely by

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*Portions from a paper submitted by J. C. Cooper and A. D. Hasler to Science.

fin clips (although only half of the morpholine-exposed groups were marked). Two of the groups were exposed to morpholine at 5×10^{-4} and 5×10^{-3} mg/l respectively, while one group was left unexposed. The fingerlings were exposed from a period three weeks before smolting to two weeks after smolting. The salmon were then released at the mouth of Oak Creek near the imprinting location. Some of these salmon returned as jacks in the fall of 1971; the majority of these salmon returned as adults in the fall of 1972, 18 months after the time of release. During this fall migration period, morpholine was released into Oak Creek at an approximate stream concentration of 1×10^{-4} mg/l (in order to decoy the salmon to this stream).

Salmon were captured in the mouth of Oak Creek, paralyzed with flaxedil (2 mg/kg body weight), and restrained in a holding box. Their gills were perfused with city tap water saturated with oxygen. All city water was filtered through activated charcoal in order to eliminate chlorine. Water was run over the portion of the fish extending out of the water to cool the body. A portion of the skull over the forebrain was removed with a dental drill to permit the insertion of an electrode into the olfactory bulb. The electrodes were made from two 00 insect pins coated with Insul-X and spaced about 1 mm apart. The EEG recordings were made with a Grass Instruments Polygraph equipped with a model 7P5 preamplifier and the signals integrated with a Grass Model 7P10 integrator. Heartbeat (EKG) was used to monitor the physiological condition of the fish. Both EEG and EKG signals were recorded on magnetic tape with an FM tape recorder. A total of 14 test samples were used (Table 10). All salmon were tested with sample aliquots of 6 to 10 ml at an approximate rate of 1 ml/sec delivered via a cannula to the ipsilateral naris in a random sequence.

There were a variety of reasons for selecting the 14 chemical solutions. Different concentrations of morpholine were used to see if fish had "remembered" the imprinting chemical. Buffered solutions were used to control for pH, which is a factor in the EEG response. Phenethyl alcohol was used to determine whether another organic compound other than morpholine would elicit evoked potentials. Lake Michigan and Oak Creek water were chosen to test for the influence of water which the fish had recently experienced and to check for the presence of non-specific stimulatory products. Finally, fish were tested with a solution of NaCl (0.06 M) because this solution produced fairly constant responses and could be used as an internal standard in our experiments.

For the purpose of standardizing the response of each trial, the integration of the responses to each test sample was divided by the integration of the NaCl record as described earlier. The means of at least three trials, and in most cases four trials, are presented for each chemical solution. The responses of fish tested only once or twice are also shown. The mean responses were ranked according to magnitude and then tested for significance by the Mann-Whitney U test (Siegel, 1956). Responses that were closer than 0.03 were considered the same rank.

Table 10. Chemical solutions used in Oak Creek experiments.

Chemical Stimuli					
Morpholine	at	1.0%	0.1%	0.01%	
Morpholine	at	1.0%	0.1%	0.01%	BUFFERED
Phenethyl Alcohol	at	0.1%	0.01%		
Phenethyl Alcohol	at	0.1%	0.01%		BUFFERED

Lake Michigan Water

Oak Creek Water

Buffer (0.1 M Sodium Bicarbonate) pH 7.5

Buffer (0.1 M Sodium Bicarbonate) pH 9.5

NaCl 0.06 M

Handwash (water in which the hand has been dipped)
or l-serine

Table 11.

ELECTROENCEPHALOGRAPHIC RESPONSES (MORPHOLINE/NaCl RESPONSES) OF IMPRINTED (M) AND NON-IMPRINTED (C) COHO SALMON, *O. kisutch*. ALL THREE OR MORE TRIALS, EXCEPT *=TWO TRIALS, ( )= ONE TRIAL.

DATE	10/16	10/16	11/4	11/16	11/2	11/14	11/19	11/14	10/26	10/31
GROUP	M*	C*	C	C*	C	C	C	C	C*	M
RESPONSE	0.54	0.79	1.00	1.00	1.02	1.02	1.03	1.11	1.21	1.33
RANK	1	2	5	5	5	5	5	8	9	10

DATE	10/31	11/15	11/28	11/2	11/4	11/27	11/9	11/21	11/14	11/15
GROUP	M	M	C	M	M	M*	M	M	(M)	M
RESPONSE	1.38	1.39	1.40	1.41	1.51	1.70	1.73	2.19	2.45	6.54
RANK	12.5	12.5	12.5	12.5	15	16.5	16.5	18	19	20

C. Results

Eleven imprinted and 9 non-imprinted fish were tested by the EEG technique. The responses of these two groups to 1% morpholine were significantly different ($U=12$, $p \leq .01$) (Table 11). If the salmon tested three or more times only are used (8 imprinted and 6 non-imprinted salmon), the responses of the two groups are also significantly different ($U=3$, $p < .001$). There were no responses to the 0.1% and 0.01% concentrations of morpholine. No differences were seen in responses for the two groups of fish imprinted at two different concentrations of morpholine (5×10^{-4} and 5×10^{-5} mg/l) as juveniles. The magnitude of the response to morpholine for the 11 imprinted fish was roughly correlated with the number of salmon returning to the stream, i.e. largest responses occurred at the peak of the spawning season (Table 12, Figure 5). The fish at the start and the end of the season showed approximately equal responses of a lower amplitude. No responses to phenethyl alcohol were observed.

Unexpectedly, we found no significant responses to the buffered morpholine solutions, although there was a significant response to pH 7.5 buffer alone (signed rank test, 44, $N=12$, $p=.003$). Thus, we have two stimuli (morpholine and pH 7.5 buffer) that individually elicit strong responses, but together show no response. Therefore, we hypothesized that the two stimuli had effects that cancelled each other out. These results cannot be explained on the basis of pH alone. One percent morpholine has a pH of 9.5, while the buffer has a pH of 7.5. Previous studies in our laboratory (Dizon, 1971) showed low responses to a pH 9.5 buffer. This result was confirmed in our experiments with buffer at pH 9.5. The magnitude of the response to the buffer solutions was random for the morpholine-exposed and control groups (Mann-Whitney U test, $U=37$, $p > .05$). However, salmon that had been previously exposed to morpholine (as juveniles) showed an increasing responsiveness to buffer during the season. Thus, this sensitization is not specific only to morpholine (the home odor). Significant differences were observed in the responses of the exposed and unexposed groups of salmon to phenethyl alcohol buffered to pH 7.5 then tested by the Mann-Whitney U test ($U=26$, $p=.05$) (Table 13).

Three qualitative differences appeared in the evoked potentials to morpholine as compared to the responses to other substances (Figure 4). First, a lag occurs for up to several seconds from the time of the stimulus until the response. This lag does not occur with buffer, handwash (water in which the hand has been dipped), phenethyl alcohol, or 0.06 M NaCl. Second, the adaptation to morpholine is very slow with clear responses lasting over 30 seconds. This compares to an adaptation time less than 10 seconds for other stimuli. Third, responses to morpholine cannot be eliminated by rinsing the naris with distilled water as is possible with other stimuli.

D. Thresholds

Although fish were imprinted to 5×10^{-4} and 5×10^{-5} mg/l concentrations of morpholine, evoked potentials occur only above the 0.01%

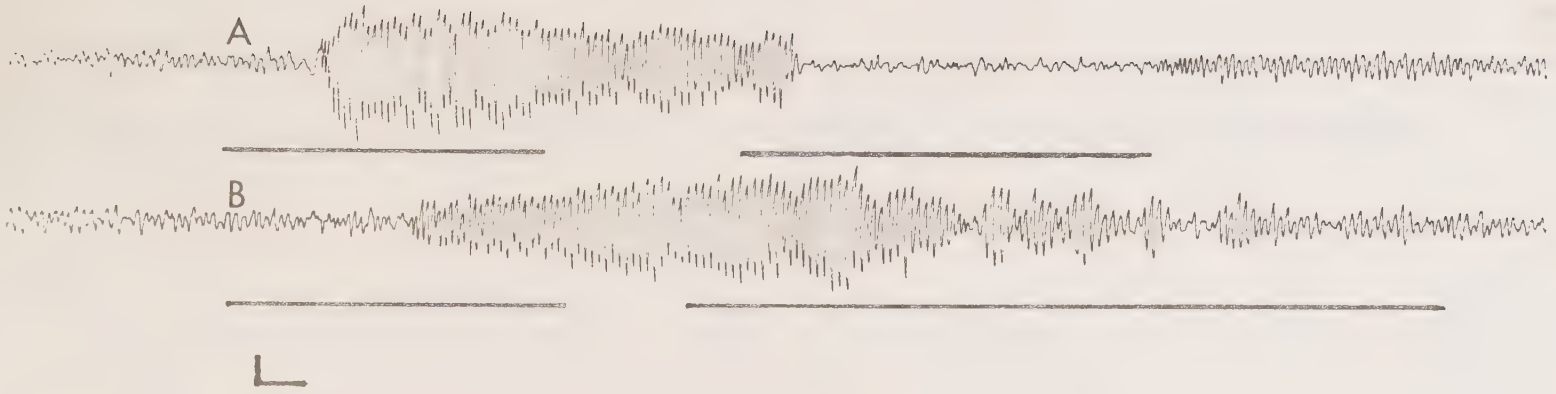

Fig. 4. A. EEG record of a response to 7.5 pH buffer. B. EEG record of a response to 1% morpholine. In each case the first stimulus marker indicates the sample and the second, a tap water rinse. The calibration mark shows one second, 200 microvolts. The morpholine response is characterized by a long lag period and a continuation of the response after a tap water rinse (Cooper).

Table 12.
RESPONSES (MORPHOLINE/NaCl) OF IMPRINTED COHO SALMON TO MORPHOLINE OVER TIME

DATE	10/16*	10/31	10/31	11/2	11/4	11/4	(11/14)	11/15	11/15	11/21	11/27*
RESPONSE	.54	1.33	1.38	1.41	1.51	1.73	2.45	1.39	6.54	2.19	1.70

Table 13. Responses to Phenethyl alcohol (buffered). Differences in responses for the morpholine exposed (M) and the unexposed groups (C) are significant (U=22, p=.05).

Date	11/28	10/16	10/16	11/16	11/14	11/16	11/27	11/9	11/14	10/31	11/14	10/31	11/19	11/2	11/4	11/4	11/21	11/2	11/15	11/15
Group	C	C*	M*	C*	C	C*	M*	M	(M)	M	C	M	C	C	M	C	M	M	M	M
Responses	.37	.72	.86	.94	.95	.94	1.00	1.00	1.00	1.03	1.06	1.08	1.12	1.18	1.29	1.48	1.58	1.81	2.34	7.19
Rank	1	2	3	5	5	5	9.5	9.5	9.5	9.5	9.5	9.5	13	14	15	16	17	18	14	20

concentration (10^2mg/l) for Dizon's work and at the 1% concentration (10^4mg/l) for this work. Table 14 shows a comparison of thresholds obtained with the EEG technique as compared to thresholds obtained by behavioral methods. It is evident that the EEG technique is the less sensitive of the two methods. One may postulate that the insensitivity of the EEG technique necessitates the use of much stronger morpholine concentrations than were needed for the initial imprinting and for decoying the fish back to Oak Creek.

Rappoport and Daginawala (1968) provide evidence that morpholine is a true olfactory stimulus, rather than an irritant. They showed that changes in base ratio nuclear RNA of the brain of the catfish were induced in one hour in sea water containing 10^{-4} M morpholine (about 10 mg/l). These changes in brain nuclear RNA were reversed within 24 hours to the levels of unexposed controls. Morpholine also elicited a change similar to other stimulants. Olfactory stimulants affect a change in content and character of the RNA in brain nuclei, whereas irritants to the olfactory epithelium change the content of brain nuclear RNA but do not alter the base ratio.

E. Short and Long-term Odor Information

The trend towards increasing responsiveness of the olfactory system to morpholine as shown by the EEG of sexually mature salmon correlated well with (a) the increasing number of fish returning to the stream [the largest evoked potentials occurred at the peak of the spawning season (Madison et al., 1973b)], (b) the stronger tendency for initial homeward orientation during the peak of the season [as shown by ultrasonic tracking studies (Madison et al., 1973a)], and (c) the absence of evoked potentials to waters that the fish had recently experienced [such responses have been reported for spawning chinook salmon (Oshima et al., 1969b)](Figure 5).

These correlations suggest that the fish can store odor information in a long-term memory system. Consciousness is not implied in our use of the term "memory." This long-term memory could be retrieved at the time of the spawning migration, perhaps facilitated by an increase in hormonal titres in the blood. At the same time, recent stimuli [e.g. the water, such as Oak Creek and Lake Michigan or artificial odors (Madison et al., 1973a) to which the fish has been recently exposed] might become less important, as shown by the lack of EEG responses to these waters.

The retention and retrieval of long-term memory traces are dynamic processes, as shown by Oshima et al. (1969c). This dynamic process is essential to the retention of the long-term memory, since it is observed that memory traces can be forgotten. Whether one holds to the school that proposes interference from other learning or to temporal decay (Gleitman and Rozin, 1971) it is vital for salmon survival that extinction of the homestream odor be counteracted by some physiological means.

In a sense, then, short-term memory may be in constant competition with the long-term memory already in existence. For the salmon with a limited brain capacity and integration areas, a mechanism that inhibits short-term memory processes for the benefit of long-term memory retrieval

Table14. Threshold from behavioral and EEG techniques to various compound (mg/l). The EEG is the less sensitive of the two.

Substance	Behavior	Worker	EEG	Worker
Morpholine	10^{-6}	Wisby (1952)	10^{4}	Cooper
			10^{2}	Dizon (1971) Dizon et. al. (1973)
			0.9	Sutterlin & Sutterlin (1971)
l-serine	10^{-4}	Brett (1954) et. al.	0.1	Hara (1972)
		Alderdice (1954) et. al.		
		Idler (1956) et. al.		
Phenethyl alcohol	10^{-3}	Teichmann (1959)	10	Cooper

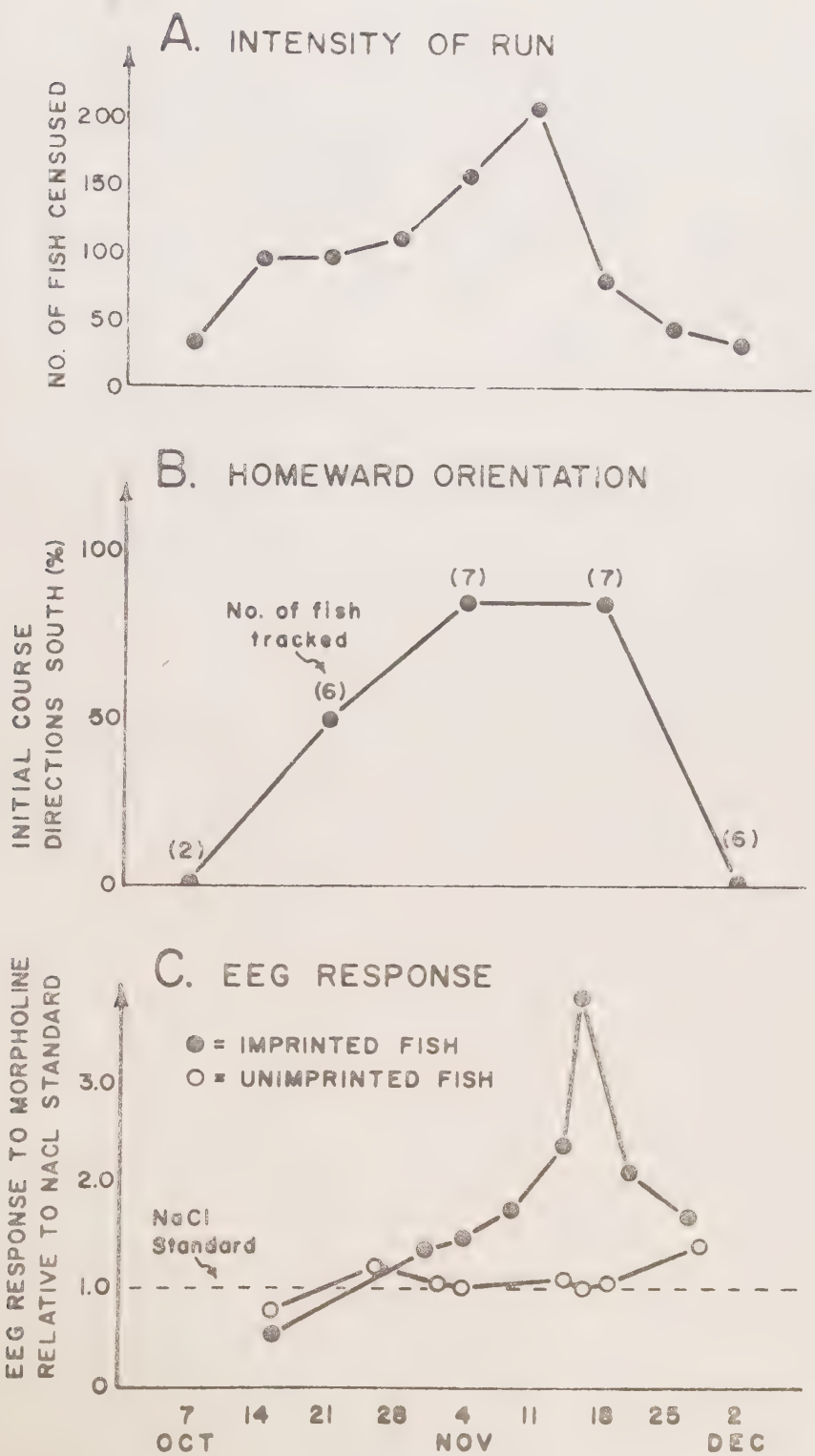

Fig. 5. Correlation of census, ultrasonic tracking and EEG studies.

might be necessary if the retrieval of the long-term memory is critical to the perpetuation of the species (Gleitman and Rozin, 1971).

There may be, for salmon, a substitution or inhibition of recent cues by the retained information (learned by imprinting) about the stream odor. This mechanism would imply that short-term odor memory is not necessary to locate the spawning site or to spawn in the home-stream and is unnecessary for survival through the spawning activities. Although there is little experimental evidence to support the above hypothesis, it is well known that salmon stop feeding as they approach the homestream. The feeding behavior may depend in part on short-term memory, (on the other hand, the relatively small contribution of short-term memory to the homing process as proposed by this hypothesis does not rule out the utilization of certain biological substances such as pheromones or fright substances, i.e. schrechstoff).

Although a behavioral and physiological preference for morpholine does not require a selective memory retrieval, it would seem non-adaptive to store information that is not required for homing.

One cannot discard the possibility that other mechanisms function in the formation of short and long-term memory. First, before the memory is consolidated, there may be an inhibition of the action short-term memory on behavior. Second, there may be a mechanism that leads to an inability to consolidate short-term memory into long-term memory, a consolidation that is an essential step as shown by Agronoff and Davis (1968). Finally, there may be an inhibition of all long-term memory retrieval except that dealing with the home odor. Whatever the mechanism, long-term memory seems to be more important than short-term memory during the homing process. Such a mechanism would have the adaptive significance of decreasing the chance of straying to non-home waters as a result of the relative insignificance of recent exposure to environmental cues. This would permit more accurate homing and thus, would tend to insure the preservation of the species.

V. SUMMARY

1. The EEG technique is not an entirely reliable method for differentiating between salmon from different homestreams. This is because there does not appear to be a unique response to a specific homestream water.

2. The analysis of the EEG is complicated by responses resulting from recent exposure to odors and by responses to non-specific stimulatory products.

3. The EEG responses are not always directly related to behavioral responses. There may be strong EEG responses and no behavioral responses to a given substance.

4. The EEG technique is useful for detecting differences in responses to specific substances, i.e. the presence or absence of morpholine in the past history of salmon. However, it is often necessary to test with concentrations far exceeding behavioral thresholds.

5. The EEG technique has been used to show that salmon can respond to morpholine 18 months from the time of last exposure to this chemical. Therefore, we hypothesize a long-term memory of this odor.

6. The EEG results did correlate well with the results from census experiments and from behavioral experiments with morpholine.

7. There may be, for salmon, a substitution or inhibition of recent cues by the retained information - long-term memory - (learned by imprinting) about the stream odor.

8. There appears to be a build-up in the magnitude of the evoked potential to morpholine towards the peak of the spawning season, indicating an increased importance of long-term memory at this time.

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